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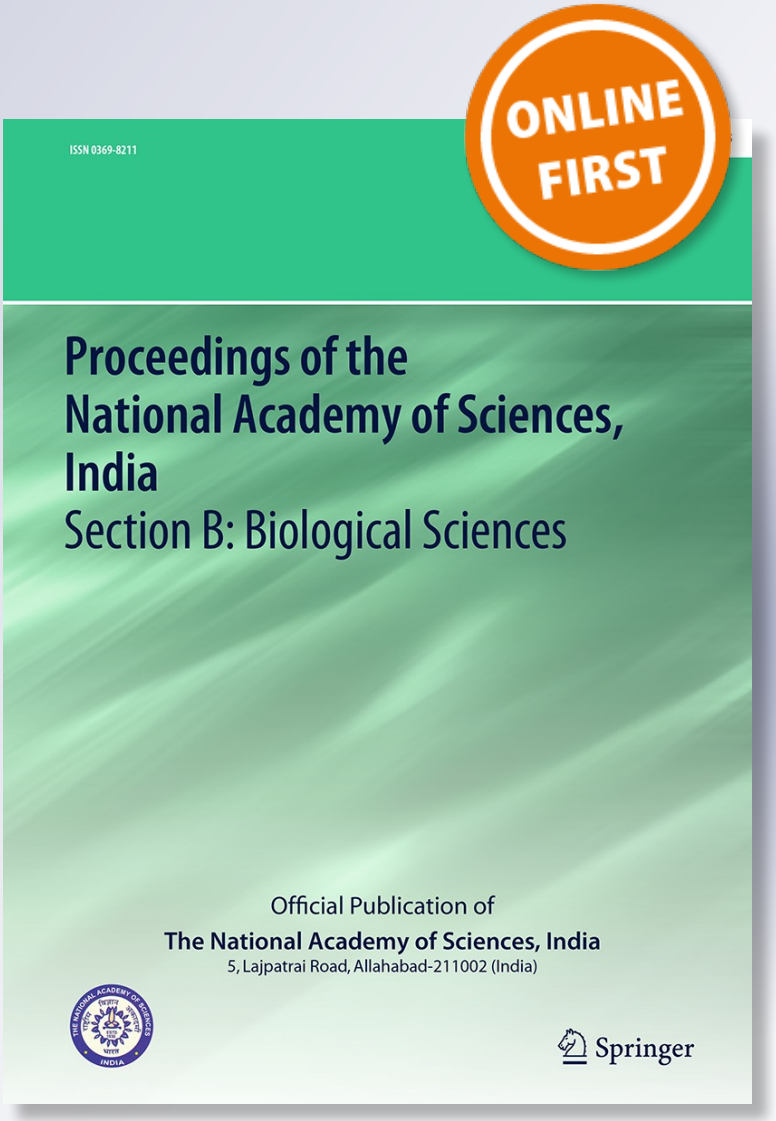

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RESEARCH ARTICLE

Effect of Indole-3-Butyric Acid on Clonal Propagation of Mulberry (*Morus alba* L.) Stem Cuttings: Rooting and Associated Biochemical Changes

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Abstract Fuel wood scarcity in the Himalayan region is an established fact and mulberry (*Morus alba* L.) tree has a great potential in fuel and energy production. This study determines the role of indole-3-butyric acid (IBA) for rapid clonal propagation of mulberry for higher biomass and large-scale production; and examines the associated biochemical changes during rooting. The non-treated (control) and treated (1000, 2000 and 3000 IBA mg L^{-1}) soft stem cuttings were cultured in mist chamber. After 50 days the rooting percentage, root number and root length were found to be higher in IBA-treated cuttings than in the non-treated ones. The rooting zone of IBA-treated and untreated cuttings were sampled at day 0 (prior to the culture in mist chamber), 15, 30, and 45 for estimation of total soluble indole content, peroxidase (POX), indole acetic acid oxidase (IAA-oxidase) and total soluble sugar (TSS). The total soluble indole, POX and IAA-oxidase were enhanced due to IBA. POX increased from day zero to day 45 of culture. IAA-oxidase kept increasing for 30 days and thereafter declined markedly. IBA initially increased TSS, which later decreased with passage of time till the 30th day both in IBA-treated and control cuttings. Thereafter, TSS content exhibited statistically non-significant variation at day 30 and 45 of culture. On the whole, IBA increased rooting phenomenon and activated carbohydrate metabolism. IAA-oxidase appears to trigger and initiate root primordia, whereas POX is involved in both initiation and elongation of roots.

Keywords Carbohydrate · Enzymatic activity · Rooting initiation · Stem cuttings

Introduction

The fodder and fuel wood scarcity in the Himalayan region is well recognized. The rural populations in the Himalayan region have long been using forest resources for their livelihood. The extreme and uncontrolled use of fodder and fuel wood has ended up with deforestation. In Garhwal Himalaya, about 77.4 % of the total human population is rural [1]. The foliage of tree forms the key source of fodder during dry months, as most of the region is rain-fed and no green fodder is grown in agricultural fields [2]. Due to poor connectivity with urban areas, the alternative sources of fuel wood are not easily accessible, thus making the population depend totally on local wood resources [3]. The biomass (fuel wood and fodder) extraction is, therefore, the major cause for vegetation depletions [4]. Most of the fodder and fuel wood tree species of Garhwal Himalayas are under stress because of an unplanned or unscientific lopping [2], whereas improvement and preservation of life in the region depend largely on forests, especially for fuel wood [5].

Morus alba L. (mulberry) is a fast-growing shrub or moderate-sized tree with a fairly cylindrical, straight bole, up to 35 m high and 1.8 m wide. It produces large quantity of renewable biomass in the form of branches, shoots, leaves and fruits. A mulberry garden yields up to 20–30

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tonnes of green leaf and 4 tonnes of mulberry sticks per hectare per year. Mulberry wood releases 4600 calories per kg, and the energy generated per hectare is 28,000 kcal [6]. In addition, mulberry is characterized as a highly valued multipurpose tree species that is used for silkworm rearing, cattle fodder, furniture making and in pharmaceutical industries. In the tropical and sub-tropical mountainous regions of the Garhwal Himalayas, mulberry is popular fuel-wood species. Now obliterated from the forest due to excess exploitation, it occurs in small patches largely cultivated around homesteads in the outer hills [2]. Having the highest total biomass and chlorophyll fluorescence (the maximum quantum yield, F_V/F_M), it was followed by *Ficus glomerata, Leucaena leucocephala* and *Ficus racemosa* at 640 m altitude [2].

Adventitious root formation in stem cuttings is a crucial physiological process for clonal propagation of many plant species. In spite of a thorough control of environmental factors in the modern propagation industry, high economic losses still occur as the result of insufficient rooting. Usually auxin, indole-3-butyric acid: IBA, and α-naphthalene acetic acid: NAA are recommended for promoting adventitious roots in stem-cutting propagation of many shrubs [7–10] and trees [11–15]. Application of auxin to cuttings causes metabolic changes during the adventitious root formation [13, 16–21], which consists of three successive but independent phases, namely induction, initiation and expression. The induction phase comprises of molecular and biochemical events without visible changes, the initiation phase is characterized by cell divisions and root-primordia organization, and the expression phase denotes the intra-stem growth of root primordia and the emergence of roots. Since rooting is a high-energy-demanding process, rooting ability of cuttings has been frequently discussed in relation to soluble and storage carbohydrate contents. Auxin also helps in mobilization of carbohydrates in leaves and upper stem, and accelerates their transport to the rooting zone [16, 17, 22, 23]. The oxidative enzymes, widely distributed in higher plants, have special significance during the rooting. Changes in the pattern of IAA-oxidase and peroxidase (POX) activities have been proposed as being the biochemical markers for the successive rooting phases [16–18, 24–26].

Given the above, effort has been made in the present study (i) to develop a rapid clonal propagation technique, using soft stem cuttings of *Morus alba*, for multiplication of superior and healthy clonal stock for a large-scale biomass production, and (ii) to understand the role of IBA in adventitious rooting of stem cuttings and accompanying biochemical changes.

Material and Methods

Plant Material and IBA Treatment

Healthy shoots of *Morus alba* tree growing in wild condition and showing superior phenotypes (height 4.58 m, GBH 0.62 m, clear and straight bole 1.65 m, and clear crown area 3.78 m) were collected in April and made into soft stem cuttings. These cuttings were dipped in 0.1 % aqueous bavistin (fungicide, BASF India Ltd., Mumbai) for 10 min and subsequently washed with distilled water. The different IBA (HiMedia Laboratory Pvt. Ltd., Mumbai, India) solutions were prepared by dissolving the appropriate amount of IBA in 50 ml of 70 % alcohol, and using distilled water to bring the solution to 100 ml. The prepared IBA solutions were stored at 4 °C in opaque bottles and used on the same day. The basal end of cuttings (~2 cm) obtained from donor plant were prepared by quick-dip method (2–3 s). The cuttings were treated with distilled water (control) or IBA (1000, 2000 and 3000 mg L^{-1}).

Culture Conditions and Experimental Design

Cuttings were planted into sterilized vermiculite (pH 7.0) pre-soaked in water for 24 h, and then cultured in trays inside a mist chamber at 32/26 °C (day/night) and 85 ± 2 % RH. The automatic day/night misting cycle was set at 60/30 s, with 1 h gap between successive cycles. A randomized complete block design was employed. There were five replications of 25 cuttings per IBA (1000, 2000 and 3000 mg IBA L^{-1}) treatment.

Sampling for data on Rooting and Biochemical Analysis

After 50 days, the cuttings were carefully removed from the rooting medium; and rooting percentage, number of roots and their length (at least one root greater than 0.50 cm in length) were recorded for each treatment. Samples from basal portions (rooting zone ~0.5 cm) were collected at day 0 (prior to the culture in mist chamber), 15, 30, and 45 for estimation of endogenous total indole (auxin) content, activity of peroxidase (POX) and indole acetic acid oxidase (IAA-oxidase), and the total soluble sugar (TSS). There were five replications with ten cutting samples per replicate. Each replicate contained five composite samples such that two cutting segments were combined together.

Table 1 Effect of auxin treatments on rooting of soft stem cuttings in *Morus alba*

IBA treatments (mg L^{-1})	Rooting parameters		
	% rooting	Roots number	Root length (cm)
Control	60.80 ± 1.36a	3.06 ± 0.28a	2.51 ± 0.21a
IBA 1000	70.20 ± 0.48b (15 %)	5.33 ± 0.18b (74 %)	4.02 ± 0.27b (60 %)
IBA 2000	76.40 ± 0.67c (25 %)	8.62 ± 0.17c (181 %)	5.25 ± 0.20b (109 %)
IBA 3000	87.00 ± 0.74d (43 %)	10.62 ± 0.32d (247 %)	6.74 ± 0.24c (168 %)

Values followed by the same letter indicate no significant differences at $P < 0.05$ level according to the Tukey's test. Each value represents the mean ± SE of five replicates. Values within parenthesis are percent variation and obtained from control

Estimation of Total Soluble IAA Content

Following the method of Donate-Correa et al. [27], one gram of sample was weighed two times; sample A was extracted with 5.0 ml of 35 % perchloric acid and sample B with 5.0 ml of modified Salkowski reagent, and kept in the dark for 1 h. Thereafter, the solutions were centrifuged at 10,000 rpm for 15 min and the supernatant was collected. Optical density (OD) of the solution was recorded at 530 nm, using a Perkin Elmer Lambda 25 UV–Vis Spectrophotometer. The relative auxin content was determined by subtracting the excitation of adjustment A from extinction of adjusted B, using the IAA standard curve, and expressed as μg g^{-1} FW.

Indole Acetic Acid Oxidase (IAA-Oxidase) Activity

IAA-oxidase activity was determined by the method of Liu et al. [28]. Reaction mixture containing 0.2 ml of enzyme extract, 0.78 ml of 50 mM potassium phosphate buffer (pH 6), 0.01 ml of 5 mM $MnCl_2$, 0.01 ml of 5 mM 2,4 dichlorophenol, 50 μg of IAA was kept for 30 min at 37 °C in the dark. The reaction was terminated with 2 ml of Salkowski reagent. The destruction of IAA was determined by measuring OD at the reaction mixture at 535 nm after 30 min, using the above mentioned spectrophotometer. The amount of IAA-oxidase activity was expressed by the amount of oxidized IAA measured in μg h^{-1} g^{-1} tissue of the rooting zone.

Peroxidase (POX) Activity

POX activity was measured using guaiacol as the substrate, as described by Husen [17]. The assay mixture contained 0.1 M phosphate buffer (pH 6.1), 4 mM guaiacol, 3 mM H_2O_2 and 0.4 ml of crude enzyme extract. The total reaction volume was 1.2 ml. OD was measured at 420 nm using spectrophotometer, and levels of enzyme activity were expressed as μmoles H_2O_2 destroyed min^{-1} mg^{-1} protein.

Total Soluble Sugar (TSS) Analysis

Extract of cutting tissues were prepared for TSS, according to Sawhney et al. [29]. The TSS content was then estimated by the phenol–sulfuric-acid method of Dubois et al. [30] and expressed in mg g^{-1} DW.

Statistical Analysis

The data on the effect IBA treatments (1000, 2000 and 3000 mg L^{-1}) on rooting response were subjected to one factor ANOVA. The data obtained for endogenous IAA content, POX, IAA-oxidase and TSS were subjected to two-factor ANOVA, i.e. IBA treatments and number of day of culture. All the means were compared by using Tukey's test at significance level $P < 0.05$ (the same letters indicate that means within a row or column are not significantly different at $P < 0.05$ level). The SPSS/PC software Ver. 16.0 was used to process the data.

Results and Discussion

Auxin regulates various aspects of plant growth and development by affecting numerous processes including cell division, cell enlargement and differentiation [31, 32]. In the present study, stem cuttings obtained from *M. alba* develop adventitious roots within 3–4 weeks after planting. There was a significant variation in rooting response between IBA treatments and the control (Table 1). After 50 days, 3000 mg IBA L^{-1} induced the maximum rooting (87 %), followed by 2000 mg IBA L^{-1} (76 %) and 1000 mg IBA L^{-1} (70 %). Further, 3000 mg IBA L^{-1} also enhanced the number of roots manifold as compared to the control. In terms of percentage, number of roots increased by 74, 181 and 247 % with 1000, 2000 and 3000 mg IBA L^{-1}, respectively. A maximum 6.74 cm root length was observed with 3000 mg IBA L^{-1}. In comparison to the control, root length increased by 60, 109 and 168 % with 1000, 2000 and 3000 mg IBA L^{-1}, respectively (Table 1).

Exogenous application of IBA to stem cuttings enhances rooting response, as reported earlier also [10, 14, 17, 33]. However, the response of rooting parameters varied with concentration of IBA; high concentration (3000 mg IBA L^{-1} in this case) was most effective in terms of rooting percentage, number of roots and their length. Induction of more roots with higher IBA concentration has been reported in various plants [10, 16, 34], but reports on the mechanism of auxin response are contradictory [32, 35]. The difference among various concentrations of IBA could be related to such factors as higher stability and slow rate of conjugation; so that higher IBA concentration is required to induce more rooting; and this will be available over a longer period of time. The present findings also suggest that exogenous application of 3000 mg IBA L^{-1} could be very effective in rapid and enhanced vegetative propagation of elite clones *M. alba* for higher biomass production. The total biomass and chlorophyll fluorescence (the maximum quantum yield, F_V/F_M) in *M. alba* have been recorded to be higher than in many other tree species [2].

Variation in rooting response due to IBA treatments was reflected in the endogenous total soluble IAA level, IAA-oxidase and peroxidase activity, and TSS content during the adventitious root primordia development. The endogenous total soluble IAA level in the rooting zone of cuttings varied significantly with IBA treatments and number of days of culture. The total soluble IAA level increased up to 15 days and declined thereafter. Exogenous IBA treatments increased the total soluble IAA as compared to the control. Thus, control cuttings showed the lowest total soluble IAA level, while the highest occurred with 3000 mg IBA L^{-1} on the 30th and 45th day of culture. At the 45th day, untreated control (13.72 μg g^{-1}-FW ± 0.86 SE), exogenous application of 1000 mg IBA L^{-1} (13.84 μg g^{-1}FW ± 0.79) and 2000 mg IBA L^{-1} (14.88 μg g^{-1}FW ± 0.83 SE) exhibited insignificant variation for total soluble IAA level. However, soluble IAA level was found to be higher (16.01 μg g^{-1}FW ± 0.65 SE) in cuttings treated with 3000 mg IBA L^{-1} up to 45 day of culture (Fig. 1a).

IAA-oxidase activity in the rooting zone of cuttings significantly varied with IBA treatments and the day of culture. It showed only a slight change till 15 days but clearly increased thereafter to attain the peak on day 30. Further, on the 45th day, it declined again. The minimum (17.41 μg h^{-1} g^{-1} ± 0.98 SE) activity appeared in the untreated control cuttings at day 0 of culture, while the maximum (29.98 μg $h^{-1}g^{-1}$ ± 1.43 SE) occurred in 3000 mg IBA L^{-1} treated cuttings on the 30th day of culture (Fig. 1b). Likewise, POX activity in the rooting zone of cuttings varied significantly with IBA treatments and the day of culture. IBA treatments had a stimulatory

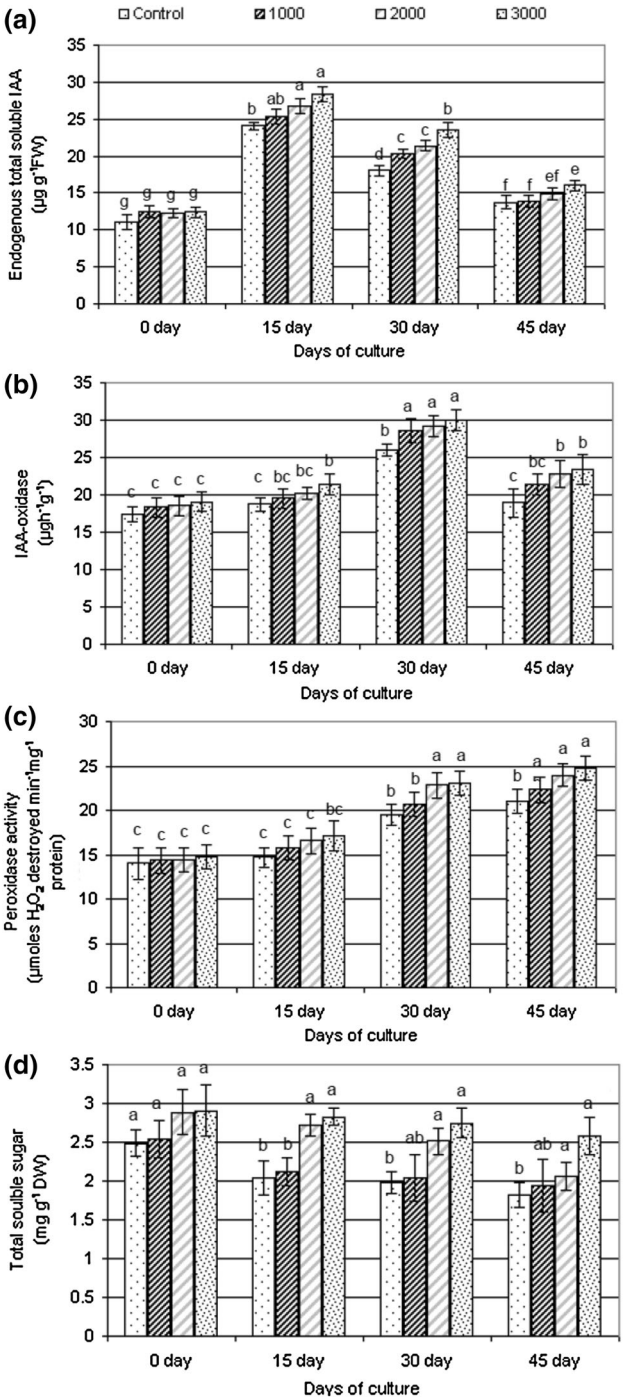

Fig. 1 Changes in **a** endogenous total soluble IAA, **b** IAA-oxidase, **c** peroxidase activity and **d** total soluble sugar in the rooting zone of *Morus alba* cuttings as affected by IBA treatments and number of days of culture. Values followed by the *same letter* indicate no significant differences at $P < 0.05$ level according to the Tukey's test. Each value represents the mean ± SE of five replicates

effect on POX activity. Like the IAA-oxidase activity, this too did not change much for 15 days, but increased significantly thereafter, and this trend was maintained up to 45 days, when the activity attained the maximum.

However, on the 45th day of culture, 3000 mg IBA L^{-1} treatment exhibited the highest (24.80 µmoles H_2O_2 destroyed min^{-1} mg^{-1} protein ± 1.35 SE) activity, while it was lowest (20.99 µmoles H_2O_2 destroyed $min^{-1}mg^{-1}$ protein ± 1.37 SE) in the untreated control (Fig. 1c).

The TSS content in the rooting zone of cuttings also varied significantly with IBA treatments and culture duration. It decreased with time up to the 30th day of culture, after which it did not show much variation. In IBA-treated samples TSS level increased up to 30 days; and did not change much beyond that. However, on the 30th day of culture, 3000 mg IBA L^{-1} treatment caused more (2.75 mg g^{-1} DW ± 0.19 SE) TSS as compared to the control (1.98 mg g^{-1} DW ± 0.14 SE). In general, from day 0 to days 30 of culture, TSS content declined in IBA-treated as well as control cuttings. The minor variations noticed later on the 30th and 45th day of culture were not statistically significant (Fig. 1d).

In general, with passage of time, the variables studied go higher in the rooting zone of IBA-treated cuttings. These findings suggest that enzymes IAA-oxidase and POX help in auxin catabolism and in triggering the root initiation processes, the former is basically having a role in triggering and initiating the root primordium, while the latter in both the initiation and elongation processes. Thus, the results provide a good linkage of these enzymes with auxin/indole metabolism and rooting phenomenon and show an involvement of oxidation products of the auxin/indole catabolism in the rooting process, and thus endorse some earlier findings [13, 16, 17]. Besides, TSS content was more at the time of planting the cuttings, and declined later during the adventitious root initiation and elongation processes. However, it remained higher in IBA-treated cuttings in comparison to the control. These results seem to substantiate the earlier reports that auxin application to the base of stem cuttings of other plant species enhanced sugar level in the rooting zone [21, 36]. Ahkami et al. [19] have proposed that applied auxin may enhance auxin concentration in the rooting zone to much higher levels so that the balance between responses at sink-establishment level (modifying the carbohydrate influx) versus root-development level (modifying the carbohydrate utilization) differs. However, enhanced sugar level in the rooting zone of cuttings caused by auxin treatments may be attributed to increase in starch hydrolysis [16, 17, 22, 37] and/or to increased sugar transport towards the rooting zone [16, 22, 38]. It is established that auxin-carbohydrate relations are vital for rooting [11, 16, 17, 39, 40].

In conclusion, the findings of this investigation suggest that 3000 mg IBA L^{-1} is the most effective IBA dose for evoking the highest rooting response. This could be gainfully used for propagation/and multiplication of mulberry stock for higher biomass production in suitable areas. Further, exogenous application of IBA induces changes in enzyme activities and in their cofactor contents, allowing for the establishment of a favourable hormone/energy balance required for root-primordium initiation and development.

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